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I, KIM MARSHALL, MANAGER EXAMINATION SUPPORT AND SALES, hereby certify that the annexed is a true copy of the Provisional specification in connection with Application No. PP 0202 for a patent by THE UNIVERSITY OF MELBOURNE filed on 5 November 1997.

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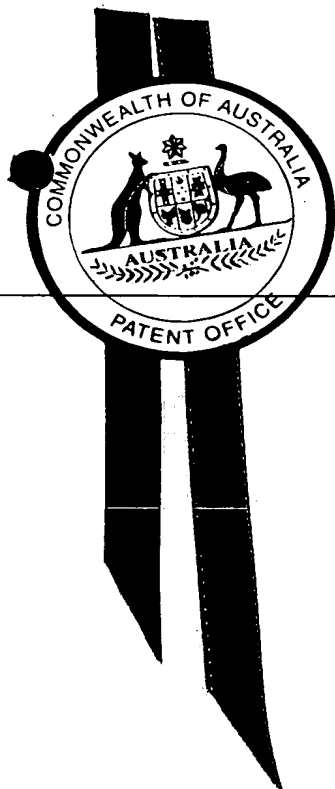
WITNESS my hand this Nineteenth
day of November 1998

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A U S T R A L I A

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"A NOVEL RECEPTOR, AND COMPOUNDS WHICH BIND THERETO"

The invention is described in the following statement:

- 1A -

A NOVEL RECEPTOR, AND COMPOUNDS WHICH BIND THERETO

The present invention relates to a novel receptor, in particular to a new type of receptor with an affinity for compounds of the oxazoline class, compounds which bind to this

5 receptor, and the use of these compounds in the treatment of diseases, especially diseases of the central nervous system, the cardiovascular system and the kidney.

The receptor according to the present invention was previously thought to be a member of the family of receptors known as imidazoline (I)-receptors. It has now been found that

10 this receptor is a separate distinct and specific site distinguishable from the known α_2- adrenoceptors and imidazoline I_1- and I_2-receptors.

Many imidazoline derivatives and structurally-related compounds including oxazolines and guanidines bind not only to α_2-adrenoceptors but also to I-receptors which differ in

15 structure, function and distribution to α_2-adrenoceptors (Parini et al, 1996, Regunathan and Reis, 1996). Based on pharmacological studies, the I-receptor binding sites (I-RBS) have been classified into I_1- and I_2-receptor subtypes: an I_1-subtype with a high affinity for [^{3}H]clonidine and an I_2-subtype with a low affinity for clonidine and a high affinity for the imidazoline radioligand, [^{3}H]idazoxan (Parini et al, 1996; Regunathan and Reis, 1996).

20

I_2-RBS have been usually labelled in tissue membranes or sections using [^{3}H]idazoxan, after masking for α_2-adrenoceptors, however, recent studies have shown that the new, high affinity radioligand [^{3}H]2-BFI labels I_2-RBS in brain and kidney, and has a greatly enhanced selectivity for I_2-RBS over α_2-adrenoceptors compared to [^{3}H]idazoxan (Hosseini et al,

25 1997; Lione et al, 1996). In rat brain the highest densities of I_2-RBS are found in the ependymal layer surrounding the ventricles, the arcuate and interpeduncular nuclei and specific nuclei of the brainstem (Mallard et al, 1995; Tesson et al, 1995). However, moderate levels of binding are also present throughout the cortex, basal ganglia, septum, hippocampus and amygdala (Mallard et al, 1995; Tesson et al, 1995). I_2-RBS have been localised to the

30 outer mitochondrial membrane and in brain appear to be associated with astrocytes specifically through an association with the mitochondrial enzyme, monoamine oxidase B

(MAO-B) (Tesson et al, 1995; Regunathan et al, 1993). A primarily glial localisation of I_2-receptors is further supported by findings that I_2-RBS are present on cultured cortical astrocytes but not neurons (Sastre and Garcia-Sevilla, 1993) and that in human brain I_2-RBS increase with age, compared to a decline in α_{2A}-adrenoceptors, consistent with known glial

5 hypertrophy and loss of cortical noradrenergic projections (Olmos et al, 1994).

A physiological function of I_2-receptors in regulating the expression of glial fibrillary acidic protein (GFAP) in astrocytes has been suggested (Alemany et al, 1995). GFAP is the main constituent of the intermediate filaments of astrocytes, which accumulate in response to brain

10 injury. A strong correlation was found between changes in GFAP immunoreactivity and changes in I_2-RBS after treatment with the imidazolines, cirazoline and idazoxan (Hosseini et al, 1997; Alemany et al, 1995), which have activity at both I_2-RBS and α_2-adrenoceptor binding sites.

15 Rilmenidine, an oxazoline, is an antihypertensive drug similar to clonidine but without its pronounced sedative effects. It has been reported as binding to both I_1- and I_2- like receptors as well as α_2-adrenoceptors (Parini et al, 1996; Evans, 1996). In some reports its non adrenergic binding has been shown to be atypical to other I compounds (King et al, 1995). However compounds of high specificity for this binding site have now been unexpectedly

20 identified, and this together with a demonstration of specific upregulation of these sites confirm it as neither an I_1- or I_2-receptor site, but as a distinct Oxazoline receptor. It has now been further established that specific I_2-receptor compounds have poor or no detectable activity at the Oxazoline receptor confirming the distinct identity of this site.

25 The specific physiological upregulation of this new receptor has been demonstrated in the rat hippocampus and its presence has been confirmed in human brain. This receptor prefers compounds of the oxazoline class and it will be herein referrred to as an oxazoline or Ox receptor.

30

Accordingly in a first aspect of the present invention there is provided a compound of the formula I:

$$ R-\underset{X}{\overset{N}{\diamond}}Y \qquad \text{I} $$
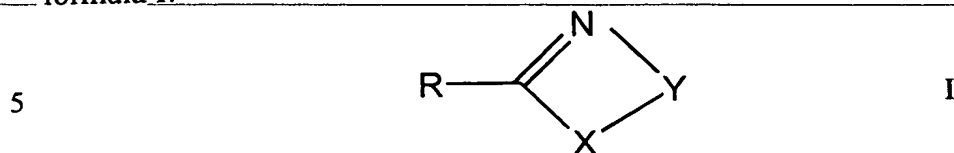
where R is the residue of an organic compound, X is O or S and Y is a divalent group making up a 5 or 6 membered ring which compound has a selectivity for the Ox receptor over one or both of the α_2- and I_2-receptors of greater than 1. Preferably R is connected to the heterocyclic ring via a divalent bridging group.

Preferably the compound of formula I has a selectivity of greater than 2, more preferably greater than 3, and most preferably greater than 5 over one or more of the α_2- and I_2- receptors.

Preferably the compound of formula I has a selectivity of greater than 1, more preferably greater than 2, over both of the α_2- and I_2-receptors.

It is also preferable that the compound of formula I has a selectivity of greater than 1, more preferably greater than 2 and most preferably greater than 3 over the I_1-receptor.

The Ox receptor is widely distributed in brain, particularly in the pineal gland, cortex, hippocampus, brain stem and spinal cord. In the hippocampus it is found in areas associated with memory, in particular the CA1-CA2 neurons i.e the pyramidal cells. In brain stem and spinal cord, the receptor is found in motor neurons and in brain stem nuclei such as the area postrema, rostroventrolateral medulla, nucleus tractus solitarius and locus coeruleus. These nuclei are associated with regulation of blood pressure. In kidney they are widely distributed in renal cortex and the outer stripe of the outer medulla, sites which are involved in regulation of ion transport.

Accordingly in a further aspect of the present invention there is provided a method for the treatment of diseases of the central nervous system, cardiovascular system or the kidney which comprises administering an effective amount of a compound of formula I or a pharmaceutically acceptable salt or ester thereof to a subject in need thereof.

5

Diseases of the central nervous system include, but are not limited to, dementia, mood disturbances, degenerative conditions, such as stroke or aging, ischaemia, CNS trauma and neurodegenerative diseases, such as Alzheimer's disease and Parkinson's disease.

10 Diseases of the cardiovascular system include, but are not limited to, hypertension and ischaemic heart disease.

Diseases of the kidney include, but are not limited to, those diseases which affect renal tubular function.

15

The compounds of formula I may also be useful in the treatment of other diseases or conditions including, but not limited to, hypoglycaemia, surgery, glaucoma and peptic ulcer, and may be useful in producing effects such as analgesia.

20 Accordingly in a further aspect of the present invention there is provided a pharmaceutical composition for the treatment of diseases of the central nervous system, the cardiovascular system or the kidney which comprises a compound of formula (I) or a pharmaceutically acceptable ester or salt thereof together with a pharmaceutically acceptable carrier or diluent.

25

The invention also provides the use of a compound of formula I in the manufacture of a medicament for the treatment of diseases of the central nervous system, the cardiovascular system or the kidney.

30 In yet another aspect of the present invention there is provided the use of a compound of formula I as an agonist or antagonist to the Ox receptor.

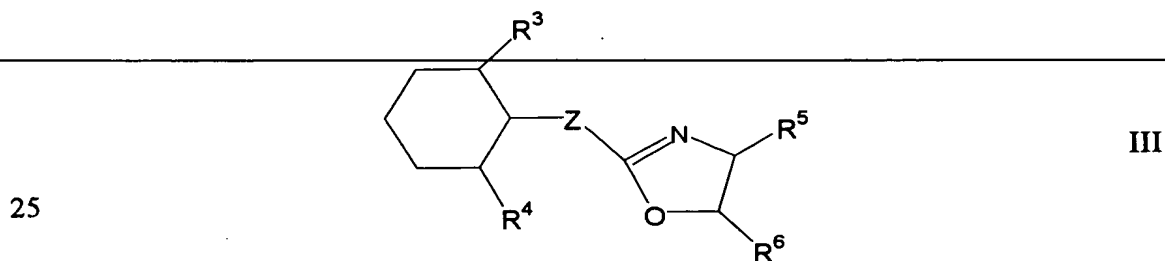In a preferred embodiment the compound of formula (I) is a compound of formula II:

II

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$$W-Z-\overset{N}{\underset{X}{\bigvee}}Y$$
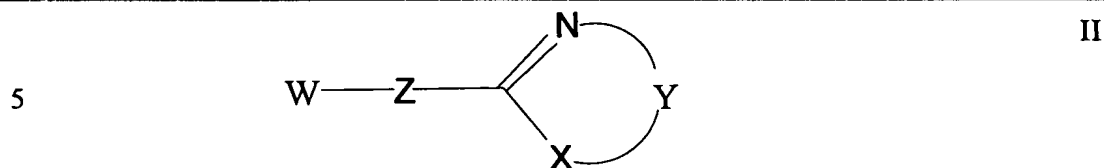
where W is selected from $-CHR^1R^2$, optionally substituted C_5-C_7 cycloalkyl, and optionally substituted aryl, where R^1 and R^2 are independently selected from hydrogen, optionally substituted C_1-C_6 alkyl and optionally substituted C_3-C_7 cycloalkyl, provided both of R^1 and R^2 are not hydrogen,

Z is imino or C_1-C_2 alkylene,

X is O or S, and

Y is optionally substituted C_2-C_4 alkylene.

In another preferred embodiment the compound of formula (I) is a compound of formula III.

III

25

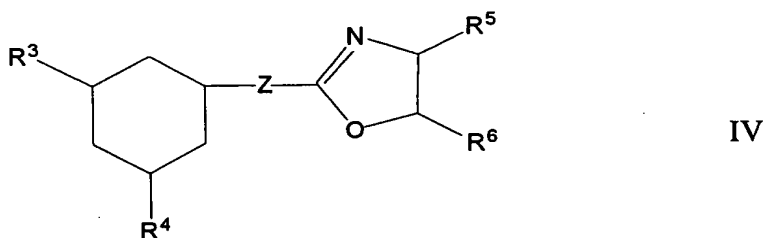wherein R^3 and R^4 are independently selected from hydrogen, C_1-C_4 alkyl and C_3-C_5 cycloalkyl,

Z is $-CH_2-$ or $-NH-$,

R^5 and R^6 are independently selected from hydrogen, C_1-C_4 alkyl, C_3-C_5 cycloalkyl, and C_3 -C_6 alkyl ester, or R^5 and R^6 may together form a 5 or 6 membered ring.

In yet another preferred embodiment the compound of formula (I) is a compound of

5 formula IV:

IV

10

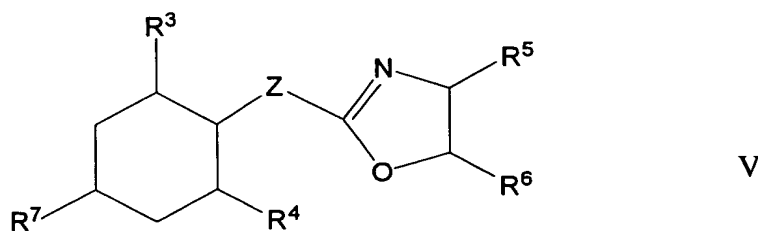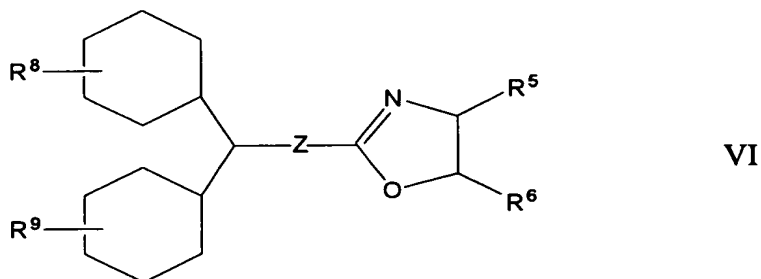where R^3, R^4, R^5, R^6 and Z are as defined above in relation to formula III.

In yet another preferred embodiment the compound of formula (I) is a compound of

15 formula V:

V

20

where R^3, R^4, R^5, R^6 and Z are as defined above in relation to formula III and R^7 is C_1-C_4 alkyl or C_1-C_4 alkoxy.

25 In yet another embodiment the compound of formula (I) is a compound of formula VI:

VI

30

where R^5, R^6 and Z are as defined above in relation to formula III and R^8 and R^9 are independently selected from hydrogen, C_1-C_6 alkyl, halogen, amino, haloalkyl, alkenyl, alkynyl, aryl, alkoxy and cycloalkyl.

5   In a further embodiment of the compound of formula (I) is a compound of formula VII:

VII

10
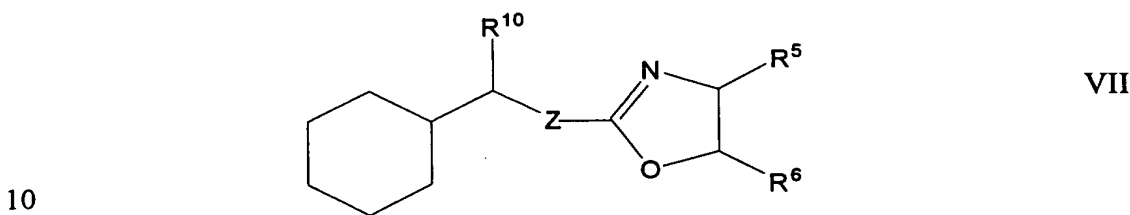
wherein R^5, R^6 and Z are as defined in relation to formula III and R^{10} is C_1-C_6 alkyl.

15   Preferably in formula III to VII Z is -NH- and R^5 and R^6 are hydrogen.

The term "aryl" as used herein refers to aromatic rings or ring systems containing between 3 and 20 carbon atoms. The rings or ring systems may be optionally substituted.

20   The term "aromatic rings or ring system" as used herein refers to a compound or substituent which includes or consists of one or more aromatic rings. The aromatic rings may be carbocyclic, heterocyclic or pseudo aromatic, and may be mono or polycyclic ring systems. Examples of suitable rings include but are not limited to benzene, biphenyl, terphenyl, quaterphenyl, naphthalene, tetradihydronaphthalene, 1-benzylnaphthalene,

25 anthracene, dihydroanthracene, benzanthracene, dibenzanthracene, phenanthracene, perylene, pyridine, 4-phenylpyridine, 3-phenylpyridine, thiophene, benzothiophene, naphthothiophene, thianthrene, furan, pyrene, isobenzofuran, chromene, xanthene, phenoxathin, pyrrole, imidazole, pyrazole, pyrazine, pyrimidine, pyridazine, indole, indolizine, isoindole, purine, quinoline, isoquinoline, phthalazine, quinoxaline,

30 quinazoline, pteridine, carbazole, carboline, phenanthridine, acridine, phenanthroline, phenazine, isothiazole, isooxazole, phenoxazine and the like, each of which may be

optionally substituted. The term "aromatic rings or ring system" includes molecules, and macromolecules, such as polymers, copolymers and dendrimers which include or consist of one or more aromatic rings. The term "pseudoaromatic" refers to a ring system which is not strictly aromatic, but which is stabilized by means of delocalization of π electrons

5   and behaves in a similar manner to aromatic rings. Examples of pseudoaromatic rings include but are not limited to furan, thiophene, pyrrole and the like.

In this specification "optionally substituted" means that a group may or may not be substituted with one or more non deleterious groups selected from alkyl, alkenyl, alkynyl,

10   aryl, halo, haloalkyl, haloalkenyl, haloalkynyl, haloaryl, hydroxy, alkoxy, alkenyloxy, aryloxy, benzyloxy, haloalkoxy, haloalkenyloxy, haloaryloxy, isocyano, cyano, formyl, carboxyl, nitro, nitroalkyl, nitroalkenyl, nitroalkynyl, nitroaryl, nitroheterocyclyl, amino, alkylamino, dialkylamino, alkenylamino, alkynylamino, arylamino, diarylamino, benzylamino, imino, alkylimine, alkenylimine, alkynylimino, arylimino, benzylimino,

15   dibenzylamino, acyl, alkenylacyl, alkynylacyl, arylacyl, acylamino, diacylamino, acyloxy, alkylsulphonyloxy, arylsulphenyloxy, heterocyclyl, heterocycloxy, heterocyclamino, haloheterocyclyl, alkylsulphenyl, arylsulphenyl, carboalkoxy, carboaryloxy mercapto, alkylthio, benzylthio, acylthio, sulphonamido, sulfanyl, sulfo and phosphorus-containing groups.

20

The term "alkyl" as used herein alone or in compound words such as "alkylester" denotes straight or branched chain alkyl, preferably C_1-C_{20} alkyl. Examples of straight chain and branched alkyl include methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, tert-butyl, amyl, isoamyl, sec-amyl, 1,2-dimethylpropyl, 1,1-dimethyl-propyl, hexyl, 4-

25   methylpentyl, 1-methylpentyl, 2-methylpentyl, 3-methylpentyl, 1,1-dimethylbutyl, 2,2-dimethylbutyl, 3,3-dimethylbutyl, 1,2-dimethylbutyl, 1,3-dimethylbutyl, 1,2,2,-trimethylpropyl, 1,1,2-trimethylpropyl, heptyl, 5-methoxyhexyl, 1-methylhexyl, 2,2-dimethylpentyl, 3,3-dimethylpentyl, 4,4-dimethylpentyl, 1,2-dimethylpentyl, 1,3-dimethylpentyl, 1,4-dimethyl-pentyl, 1,2,3,-trimethylbutyl, 1,1,2-trimethylbutyl, 1,1,3-

30   trimethylbutyl, octyl, 6-methylheptyl, 1-methylheptyl, 1,1,3,3-tetramethylbutyl, nonyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-methyl-octyl, 1-, 2-, 3-, 4- or 5-ethylheptyl, 1-, 2- or 3-

propylhexyl, decyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- and 8-methylnonyl, 1-, 2-, 3-, 4-, 5- or 6-
ethyloctyl, 1-, 2-, 3- or 4-propylheptyl, undecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8- or 9-
methyldecyl, 1-, 2-, 3-, 4-, 5-, 6- or 7-ethylnonyl, 1-, 2-, 3-, 4- or 5-propylocytl, 1-, 2-
or 3-butylheptyl, 1-pentylhexyl, dodecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7-, 8-, 9- or 10-

5 methylundecyl, 1-, 2-, 3-, 4-, 5-, 6-, 7- or 8-ethyldecyl, 1-, 2-, 3-, 4-, 5- or 6-
propylnonyl, 1-, 2-, 3- or 4-butyloctyl, 1-2-pentylheptyl and the like.

The term "cycloalkyl" as used herein denotes cyclic alkyl groups, including mono- and
polycyclic alkyl groups.   Examples of cyclic alkyl include mono- or polycyclic alkyl

10 groups such as cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, cycloheptyl, cyclooctyl,
cyclononyl, cyclodecyl and the like.   Preferably the cyclic alkyl groups are C_3-C_6 alkyl
groups such as cyclopentyl and cyclohexyl.

The term "alkoxy" denotes straight chain or branched alkoxy, preferably C_{1-20} alkoxy.

15 Examples of alkoxy include methoxy, ethoxy, n-propoxy, isopropoxy and the different
butoxy isomers.

The term "alkenyl" denotes groups formed from straight chain, branched or cyclic alkenes
including ethylenically mono-, di- or poly-unsaturated alkyl or cycloalkyl groups as

20 previously defined, preferably C_{2-20} alkenyl.   Examples of alkenyl include vinyl, allyl, 1-
methylvinyl, butenyl, iso-butenyl, 3-methyl-2-butenyl, 1-pentenyl, cyclopentenyl, 1-
methyl-cyclopentenyl, 1-hexenyl, 3-hexenyl, cyclohexenyl, 1-heptenyl, 3-heptenyl, 1-
octenyl, cyclooctenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 1-decenyl, 3-decenyl, 1,3-
butadienyl, 1-4,pentadienyl, 1,3-cyclopentadienyl, 1,3-hexadienyl, 1,4-hexadienyl, 1,3-

25 cyclohexadienyl, 1,4-cyclohexadienyl, 1,3-cycloheptadienyl, 1,3,5-cycloheptatrienyl and
1,3,5,7-cyclooctatetraenyl.

The term "alkynyl" denotes groups formed from straight chain, branched or cyclic alkyne
including alkyl and cycloalkyl groups as previously defined which contain a triple bond,

30 preferably C_{2-20} alkynyl.   Examples of alkynyl include ethynyl, 2,3-propynyl and 2,3- or
3,4-butynyl.

The term "heterocyclic" as used herein refers to aromatic, pseudo-aromatic and non-aromatic rings or ring systems which contain one or more heteroatoms selected from N, S and O and which may be optionally substituted. Preferably the rings or ring systems have

5 3 to 20 carbon atoms.

Where a carbon is asymmetrically substituted and optical isomers exist for compounds of formula I it has been found that the optical isomers vary in their affinity and selectivity for the Ox receptor and α_2-adrenoceptor's as much as 100-fold.

10

Free base forms of the compounds of the invention may be converted into the acid addition salts in a conventional manner and vice versa. Suitable acids for addition salts include, hydrochloric, tartaric, malonic and fumaric.

15 In the compounds of the invention the carbon atom next to Z may be asymmetrically substituted as may the carbons of the oxazoline ring. The compounds may be prepared as racemic mixtures or the isomers may be isolated in the conventional manner from asymmetric starting materials or by resolution of the base on fractional crystallisation with optically active acids.

20

A compound of formula I, where X is O, Y is CH_2CH_2, and R is connected to the heterocyclic ring via a bridging group -NH-, may be prepared in two steps by converting the appropriate primary amine into an oxazoline via the chloroethyl urea.
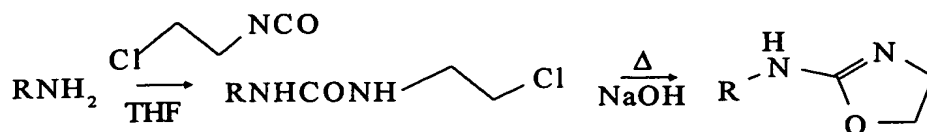
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Other compounds may be prepared in analogous manner.

Some of the compounds of formula I are novel and represent a further aspect of the present invention.

5

Some compounds of formula II and III are known and are excluded from this further aspect of the invention.

Compounds with the general formulae III and V where Z is -NH-, R^5 and R^6 are H, R^3 is

10   lower alkyl of 1 to 3 carbon atoms and R^4 and R^7 are independently H or a lower alkyl of 1 to 5 carbon atoms have been previously disclosed in US3598833 and US5034406 to be useful in the following therapeutic areas: reducing or maintaining intra-ocular pressure, for example in the treatment of glaucoma; increasing blood-sugar levels, for example in the treatment of hyperglycemia; inhibiting gastric-secretion, for example in the treatment of ulcers and

15   hypertonia; vasoconstriction; depressing blood pressure; sedation; local anesthesia and hypertension.

Compounds with the general formula II where Z is -NH-, X is O, Y is -CH_2CH_2- and W is CHR^1R^2 where R^1 and R^2 can each independently be H (but not both H), or a low molecular

20   weight alkyl group substituted with an optionally substituted cyclopropyl have been previously disclosed in US3988464 and US4102890 to be useful in the following therapeutic areas: in treating hypertension; treating heart failure and edematous disease; analgesia; depressing the cardiovascular system; neuromodulation; lowering blood pressure; treating cardiovascular diseases; treating neuropsychological disturbances; depression of the CNS

25   which may result in hypnosis, analgesic action and or antipsychotic effects.

Compounds with the general formula II where Z is -NH-, X is O, Y is -CH_2CH_2- and W is CHR^1R^2 where R^1 can be a haloalkyl of 1 to 3 carbon atoms and R^2 can be a lower alkyl radical having 1 to 6 carbon atoms in a straight or branched chain, a lower cycloalkyl radical

30   having from 3 to 7 carbon atoms which may be substituted by one or more lower alkyl radicals, or lower alkoxy radicals have been previously disclosed in US4378366 and DE4325491 to be useful in the following therapeutic areas: in treating hypertension; treating

- 12 -

acute alterations in intermediary metabolism subsequent to surgery, trauma and burns.

Compounds with the general formula II where Z is -NH-, X is O, Y is -CH$_2$CH$_2$- and W is CHR^{1}R^2 where R$_1$ can be a haloalkyl of 1 to 3 carbon atoms and R$_2$ can be an optionally

5 substituted phenyl or furanyl or thiophene or pyrrole group have been previously disclosed in US4267345 to be useful as antihypertensive agents.

The compound with the general formula II where Z is -NH-, X is O, Y is -CH$_2$CH$_2$- and W is CHR^{1}R^2 where R^1 and R^2 are cyclopropyl groups has been previously disclosed in patents

10 US5605911, US5459133 and DE4325491 to be useful in the following therapeutic areas: treating post aggression syndrome, preventing neurotoxic side-effects when administered with an NMDA antagonist; combatting a naturally occurring form of NMDA-receptor hypofunction which occurs as a causative or aggravating mechanism in people suffering from schizophrenia; reduction of at least one neurotoxic side effect selected from the group

15 consisting of formation of vacuoles in neurons in cerebrocortical or limbic brain regions, expression of heat shock proteins in cerebrocortical or limbic brain regions, alteration or loss of mitochondria in neurons, neuronal death or hallucinations and psychotomimetic effects; increasing the accommodative ability of the eye for the purpose of decreasing the severity of presbyopsia.

20

Homogenate binding studies have shown that I$_2$-RBS increase with age in human brain, consistent with glial hypertrophy. In autoradiographic studies we have compared the spatial relationship of I$_2$-RBS and Ox receptor sites and astrocytes in young and aged rats. At a level through the rostral hippocampus [^{3}H]2-BFI (a specific I$_2$-receptor ligand) binding was

25 not altered in the brain of 24 month-old rats compared to 3 month-old rats, although GFAP mRNA levels were elevated throughout both white and grey matter areas. Unexpectedly there was a dramatic difference in the pattern of Ox receptors. Binding to this receptor in the pyramidal cell layer of the CA1-CA3 region of the hippocampus was more prominent in the aged rats and there was a marked concentration of binding over the CA2 region. Additional

30 binding was evident in layer 3 of the parietal cortex, the ventromedial and dorsomedial hypothalamic nuclei and the medial amygdaloid nuclei in the aged animals. This binding

pattern distinct from that of GFAP mRNA suggests the binding is localised to neurons rather than to glial cells and provides further evidence of a distinct neuronal receptor site that is physiologically upregulated.

5   Short periods of global cerebral ischaemia induce selective neuronal loss in the CA1 field of the hippocampus in experimental models in animals and in humans after cardiac arrest (Petito et al, 1990). This delayed neuronal death has been widely studied in the rat four-vessel occlusion (4-VO) model and is accompanied by a reactive astrocytosis involving cell hypertrophy and hyperplasia and transformation of GFAP-negative into GFAP-positive glia

10 that persist for some weeks post-ischaemia (Gustafson et al, 1990). It was originally hypothesised that I_2-RBS might be linked to this altered expression of GFAP, however, it was found when examining the spatial relationship of changes in I_2-RBS and reactive gliosis following 4-VO ischaemic injury in rats the pattern of binding of the selective I_2-RBS ligand, [³H]2-BFI, was unaltered. Unexpectedly, specific binding to Ox receptors was increased by

15 approximately 60%, 4 and 8 days after the ischaemic insult but had returned to normal levels at later time points. This increase was observed only in the CA1 pyramidal cell region of the hippocampus and was not related to reactive astrocytosis. The upregulation of Ox receptors following ischaemia demonstrates for the first time that these Ox sites are involved in pathophysiological processes in the brain which appear to represent compensatory

20 mechanisms in surviving neurons and provide a rationale for use of compounds which act at these sites as neuroprotective agents. The changes in Ox receptors also provide further evidence that the Ox receptor is a novel receptor site, distinct from I_2-receptors and α_2-adrenoceptors.

25 The Ox receptor pharmacophore as depicted by formula I differs from those of the I_2-receptor (Nero et al, 1997) and α_2-adrenoceptor (Timmermans and van Zwieten, 1977) in the nature of the bridging moiety Z and the hydrogen bond donor (or acceptor) groups (-NH- and X). For both the Ox receptor and α_2-adrenoceptor pharmacophores the bridging moiety Z induces a bend into the molecular configuration, whereas in the I_2-receptor pharmacophore

30 Z, typically a bond, maintains an overall linear molecular configuration. The nature of the

hydrogen bond donor (or acceptor) groups further differentiates the Ox receptor pharmacophore from the I_2-receptor pharmacophore in that compounds containing an imidazoline ring (where X is NH) have poor (μM) affinity at the Ox receptor binding site and high (nM) affinity at the I_2-RBS. While compounds containing an oxazoline ring (X is O)

5   have high affinity (nM) for the Ox receptor binding site and poor (μM) affinity at the I_2-RBS. The Ox receptor pharmacophore can be further distinguished from the α_2-adrenoceptor pharmacophore by the substituents R_5 and R_6 as present in formula III. Substitutions in this region of the compound reduce, or abolish, α_2-adrenoceptor affinity while maintaining affinity for the Ox receptor.

10

While it is possible that, for use in therapy, compounds of the invention may be administered as the raw chemical, it is preferable to present the active ingredient as a pharmaceutical formulation.

15 The invention thus further provides pharmaceutical formulations comprising a compound of the invention or a pharmaceutically acceptable salt or derivative thereof together with one or more pharmaceutically acceptable carriers therefor and, optionally, other therapeutic and/or prophylactic ingredients. The carrier(s) must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not deleterious to the recipient thereof.

20

Pharmaceutical formulations include those suitable for oral, rectal, nasal, topical (including buccal and sub-lingual), vaginal or parenteral (including intramuscular, sub-cutaneous and intravenous) administration or in a form suitable for administration by inhalation or insufflation.

25

The compounds of the invention, together with a conventional adjuvant, carrier, or diluent, may thus be placed into the form of pharmaceutical compositions and unit dosages thereof, and in such form may be employed as solids, such as tablets or filled capsules, or liquids such as solutions, suspensions, emulsions, elixirs, or capsules filled with the same, all for

30 oral use, in the form of suppositories for rectal administration; or in the form of sterile

injectable solutions for parenteral (including subcutaneous) use. Such pharmaceutical compositions and unit dosage forms thereof may comprise conventional ingredients in conventional proportions, with or without additional active compounds or principles, and such unit dosage forms may contain any suitable effective amount of the active ingredient

5   commensurate with the intended daily dosage range to be employed. Formulations containing one (1) milligrams of active ingredient or, more broadly, 0.001 to three hundred (300) milligrams, per tablet, are accordingly suitable representative unit dosage forms.

The compounds of the present invention can be administered in a wide variety of oral and

10   parenteral dosage forms. It will be obvious to those skilled in the art that the following dosage forms may comprise, as the active component, either a compound of the invention or a pharmaceutically acceptable salt of a compound of the invention.

For preparing pharmaceutical compositions from the compounds of the present invention,

15   pharmaceutically acceptable carriers can be either solid or liquid. Solid form preparations include powders, tablets, pills, capsules, cachets, suppositories, and dispersible granules. A solid carrier can be one or more substances which may also act as diluents, flavouring agents, solubilizers, lubricants, suspending agents, binders, preservatives, tablet disintegrating agents, or an encapsulating material.

20
In powders, the carrier is a finely divided solid which is in a mixture with the finely divided active component.

In tablets, the active component is mixed with the carrier having the necessary binding

25   capacity in suitable proportions and compacted in the shape and size desired.

The powders and tablets preferably contain from five or ten to about seventy percent of the active compound. Suitable carriers are magnesium carbonate, magnesium stearate, talc, sugar, lactose, pectin, dextrin, starch, gelatin, tragacanth, methylcellulose, sodium

30   carboxymethylcellulose, a low melting wax, cocoa butter, and the like. The term

preparation" is intended to include the formulation of the active compound with
encapsulating material as carrier providing a capsule in which the active component, with or
without carriers, is surrounded by a carrier, which is thus in association with it. Similarly,
cachets and lozenges are included. Tablets, powders, capsules, pills, cachets, and lozenges

5   can be used as solid forms suitable for oral administration.

For preparing suppositories, a low melting wax, such as admixture of fatty acid glycerides or
cocoa butter, is first melted and the active component is dispersed homogeneously therein,
as by stirring. The molten homogenous mixture is then poured into convenient sized molds,

10   allowed to cool, and thereby to solidify.

Formulations suitable for vaginal administration may be presented as pessaries, tampons,
creams, gels, pastes, foams or sprays containing in addition to the active ingredient such
carriers as are known in the art to be appropriate.

15

Liquid form preparations include solutions, suspensions, and emulsions, for example, water
or water-propylene glycol solutions. For example, parenteral injection liquid preparations
can be formulated as solutions in aqueous polyethylene glycol solution.

20   The compounds according to the present invention may thus be formulated for parenteral
administration (e.g. by injection, for example bolus injection or continuous infusion) and may
be presented in unit dose form in ampoules, prefilled syringes, small volume infusion or in
multi-dose containers with an added preservative. The compositions may take such forms as
suspensions, solutions, or emulsions in oily or aqueous vehicles, and may contain

25   formulatory agents such as suspending, stabilising and/or dispersing agents. Alternatively,
the active ingredient may be in powder form, obtained by aseptic isolation of sterile solid or
by lyophilisation from solution, for constitution with a suitable vehicle, e.g. sterile, pyrogen-
free water, before use.

30   Aqueous solutions suitable for oral use can be prepared by dissolving the active component

- 17 -

in water and adding suitable colorants, flavours, stabilizing and thickening agents, as desired.

Aqueous suspensions suitable for oral use can be made by dispersing the finely divided active component in water with viscous material, such as natural or synthetic gums, resins,

5   methylcellulose, sodium carboxymethylcellulose, or other well known suspending agents.

Also included are solid form preparations which are intended to be converted, shortly before use, to liquid form preparations for oral administration. Such liquid forms include solutions, suspensions, and emulsions. These preparations may contain, in addition to the active

10   component, colorants, flavours, stabilizers, buffers, artificial and natural sweeteners, dispersants, thickeners, solubilizing agents, and the like.

For topical administration to the epidermis the compounds according to the invention may be formulated as ointments, creams or lotions, or as a transdermal patch. Ointments and

15   creams may, for example, be formulated with an aqueous or oily base with the addition of suitable thickening and/or gelling agents. Lotions may be formulated with an aqueous or oily base and will in general also contain one or more emulsifying agents, stabilising agents, dispersing agents, suspending agents, thickening agents, or colouring agents.

20   Formulations suitable for topical administration in the mouth include lozenges comprising active agent in a flavoured base, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient in an inert base such as gelatin and glycerin or sucrose and acacia; and mouthwashes comprising the active ingredient in a suitable liquid carrier.

25   Solutions or suspensions are applied directly to the nasal cavity by conventional means, for example with a dropper, pipette or spray. The formulations may be provided in single or multidose form. In the latter case of a dropper or pipette, this may be achieved by the patient administering an appropriate, predetermined volume of the solution or suspension. In the case of a spray, this may be achieved for example by means of a metering atomising spray

pump.

Administration to the respiratory tract may also be achieved by means of an aerosol formulation in which the active ingredient is provided in a pressurised pack with a suitable

5   propellant such as a chlorofluorocarbon (CFC) for example dichlorodifluoromethane, trichlorofluoromethane, or dichlorotetrafluoroethane, carbon dioxide, or other suitable gas. The aerosol may conveniently also contain a surfactant such as lecithin. The dose of drug may be controlled by provision of a metered valve.

10   Alternatively the active ingredients may be provided in the form of a dry powder, for example a powder mix of the compound in a suitable powder base such as lactose, starch, starch derivatives such as hydroxypropylmethyl cellulose and polyvinylpyrrolidone (PVP). Conveniently the powder carrier will form a gel in the nasal cavity. The powder composition may be presented in unit dose form for example in capsules or cartridges of, e.g., gelatin, or

15   blister packs from which the powder may be administered by means of an inhaler.

In formulations intended for administration to the respiratory tract, including intranasal formulations, the compound will generally have a small particle size for example of the order of 5 microns or less. Such a particle size may be obtained by means known in the art, for

20   example by micronization.

When desired, formulations adapted to give sustained release of the active ingredient may be employed.

25   The pharmaceutical preparations are preferably in unit dosage forms. In such form, the preparation is subdivided into unit doses containing appropriate quantities of the active component. The unit dosage form can be a packaged preparation, the package containing discrete quantities of preparation, such as packeted tablets, capsules, and powders in vials or ampoules. Also, the unit dosage form can be a capsule, tablet, cachet, or lozenge itself, or it

30   can be the appropriate number of any of these in packaged form.

Tablets or capsules for oral administration and liquids for intravenous administration are preferred compositions.

5   A further aspect of the present invention contemplates an isolated Ox receptor polypeptide.

Preferably, the isolated Ox receptor polypeptide is in a sequencably pure form.

10   By "sequencably pure" is meant that the purified Ox receptor is provided in a form which is sufficiently homogeneous and, if necessary, modified to facilitate amino acid sequence determination.

Standard methods may be used to isolate the Ox receptor polypeptide for example gel
15   filtration, ion-exchange chromatography, hydrophobic interaction chromatography, reverse-phase chromatography, electrophoresis, affinity chromatography or immuno-affinity chromatography, amongst others or any combination thereof. Such methods are well-known to those skilled in the art and the use of all such methods to isolate an Ox receptor polypeptide are encompassed by the present invention.

20

In a particularly preferred embodiment, the Ox receptor polypeptide is isolated using at least one affinity chromatography step, optionally in association with one or more other procedures for protein purification. As will be known to those skilled in the art, the isolation of an Ox receptor polypeptide using affinity chromatography requires a
25   biospecific ligand capable of specifically binding to the intact Ox receptor or an Ox receptor polypeptide to be attached to a chromatographic matrix for example Sepharose™, Superose™, cellulose or Sephacryl™ (Pharmacia Fine Chemicals, Uppsala, Sweden), amongst others such that the immobilised ligand retains its specific binding affinity for the Ox receptor or Ox receptor polypeptide.

- 20 -

Preferably, the biospecific ligand is a chemical compound which exhibits specific and reversible binding-affinity-for-the-intact-Ox-receptor-or-an-Ox-receptor-polypeptide.

Particularly preferred biospecific ligands contemplated by the invention include Ox
5   receptor substrate compounds and substrate analogues, such as those described herein. More preferably, the biospecific ligand is a compound having the structure set forth in formula II, even more preferably a compound having the structure set forth in Formula II, wherein X is O, Y is -CHR^{5}CHR^6 where R^5 and R^6 are H, W is -CHR^{1}R^2 where R^1 is 4-amino, 2-methoxyphenyl and R^2 is H, and Z is NH.

10

The biospecific ligand is attached to the chromatographic matrix either directly or alternatively, *via* a spacer arm placed therebetween, for example a spacer arm comprising a short alkyl group having between 1 and 12 carbon atoms, to reduce steric interference between the matrix and the Ox receptor. Standard covalent coupling procedures may be
15   employed, for example coupling via cyanogen bromide, carbodiimide, epoxy, thiopropyl, diazonium or bromoacetamidoalkyl groups, amongst others.

The Ox receptor in cellular extract or a derivative fraction thereof in a suitable "binding buffer", is applied to the affinity chromatography column under conditions sufficient to
20   promote binding of the receptor to the column. The non-specific binding of molecules other than Ox receptors may be minimised by including in the binding buffer one or more chemical compounds for which said molecules have a high binding affinity compared to the Ox receptor. Alternatively, or in addition, the binding buffer may include one or more protease inhibitors, serine protease inhibitors, trypsin inhibitors, leupeptin or PMSF,
25   to minimise proteolysis of the Ox receptor during the isolation step. Once the Ox receptor is bound to the column, the column is washed several times with binding buffer to remove contaminants.

The bound Ox receptor may be eluted from the affinity matrix using a variety of elution

methods known to those skilled in the art. Elution methods may be either selective or non-selective in nature. Usually, selective elution methods are applied in combination with group specific adsorbents and non-selective elution methods are used in combination with highly specific adsorbents. A compromise may have to be made between the

5   harshness of the eluent required for elution and the risk of denaturing the eluted material. Forces which maintain the complex include electrostatic interactions, hydrophobic effects and hydrogen-bonding. Agents which weaken these interactions may be expected to function as efficient eluting agents. Elution may be achieved by either a step-wise or a continuous change in conditions. Continuous gradients are useful for their zone

10 sharpening effect; the trailing edge of a desorbed substance experiences a higher concentration of eluting agent than the peak and is therefore made to move faster down the column than the main peak. When substances are very tightly bound to the absorbent, it may be effective to stop the flow after applying eluent and to allow the adsorbent to remain in the presence of eluting agent (30 min to 2h is commonly used) before

15 commencing elution. This allows dissociation to take place prior to elution and thus ensures good recoveries of adsorbed substances.

Changes in pH, ionic strength, polarity or the use of denaturing agents such as chaotropic salts, guanidine-HCl or urea or electrophoretic techniques may be used to desorb the

20 bound Ox receptor or Ox receptor polypeptide from the affinity matrix.

Alternatively, the Ox receptor or Ox receptor polypeptide may be desorbed using a selective eluent capable of competing for the binding of the receptor or receptor polypeptide to the ligand. The selective eluent may be applied to the column in a suitable

25 buffer composition at a single concentration or a concentration gradient of the eluent in the buffer may be used.

In a particularly preferred embodiment the Ox receptor or Ox receptor polypeptide is eluted using a selective eluent comprising O501.

- 22 -

The isolated Ox receptor polypeptide is provided in a sequencably pure form by any means of preparing proteins for amino acid sequence determination known to those skilled in the art. Preferably, the polypeptide is purified using at least one chromatographic or electrophoretic step.

5

Whilst the N-terminus of the isolated Ox receptor polypeptide may be sequenced, it is particularly desirable to obtain internal amino acid sequences of the Ox receptor polypeptide. Accordingly, a sequencably pure Ox receptor polypeptide will preferably comprise a proteolytic digestion product of the complete Ox receptor polypeptide,

10 obtained for example by digestion of the isolated polypeptide with trypsin and/or endo-lysC. As with the isolated protein, the peptides obtained by proteolytic digestion are further purified to facilitate their amino acid sequence determination.

Peptides are generally transferred to PVDF membranes to facilitate the sequence

15 determination of nanomolar quantities or even femtomolar quantities of peptide, and sequenced using Edman degradation or other techniques known to those skilled in the art.

The amino acid sequence of the Ox receptor polypeptide is useful for the preparation of degenerate pools of synthetic oligonucleotides encoding same or complementary thereto.

20

Those skilled in the art will be aware that such oligonucleotide pools are particularly useful as hybridisation probes and/or amplification primers in the isolation of nucleic acid molecules which encode the Ox receptor polypeptide described herein. In preparing oligonucleotide pools for such purposes, it is desirable to utilise those amino acid

25 sequences of peptides which include one or more amino acid selected from the list comprising phenylalanine, tryptophan, tyrosine, cysteine, histidine, glutamine, asparagine, lysine, aspartate or glutamate or alternatively, which include no more than one amino acid selected from the list comprising leucine, serine and arginine. Selection of amino acid sequences in accordance with these parameters reduced the number of different

- 23 -

oligonucleotide sequences which are required to provide a representative pool including all possible Ox receptor polypeptide-encoding sequences.

The oligonucleotides described herein will at least comprise a combination of any of the

5 nucleotides adenine, cytidine, guanine, thymidine, or inosine, or functional analogues or derivatives thereof which are at least capable of being incorporated into a polynucleotide molecule without having an inhibitory effect on their use as hybridisation probes or primer molecules.

10 Furthermore, one or both of the nucleic acid primer molecules may be contained in an aqueous mixture of other nucleic acid primer molecules, for example a mixture of degenerate primer sequences which vary from each other by one or more nucleotide substitutions or deletions. Alternatively, one or both of the nucleic acid primer molecules may be in a substantially pure form.

15

The oligonucleotide sequences derived from isolated Ox receptor peptide sequences are employed to identify and isolate Ox receptor-encoding nucleotide sequences from cells, tissues, or organ types of any mammalian species which expresses an Ox receptor, in particular a laboratory mammal such as a rat, mouse or rabbit.

20

In one approach, genomic DNA, or mRNA, or cDNA derived from the cells, tissues or organs of said mammal, in particular kidney tissue, is contacted with a hybridisation effective amount of one or more olignucleotide pools described herein or a complementary sequence and then the hybridising genomic DNA, or mRNA, or cDNA is detected.

25

In a particularly preferred embodiment, the Ox receptor-encoding oligonucleotide is end-labelled with a reporter molecule capable of giving an identifiable signal (e.g. a radioisotope such as ^{32}P or ^{35}S or a biotinylated molecule).

- 24 -

The hybridising genetic sequence thus identified is preferably in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell.

An alternative method contemplated in the present invention involves hybridising two
5   nucleic acid "primer molecules" derived from the Ox receptor peptide amino acid sequence described herein to a nucleic acid "template molecule" comprising an Ox receptor-encoding gene or a part thereof. According to this embodiment, specific nucleic acid molecule copies of the template molecule are amplified enzymatically in a polymerase chain reaction, a technique that is well known to one skilled in the art.

10

In a preferred embodiment, each nucleic acid primer molecule is at least 10 nucleotides in length, more preferably at least 20 nucleotides in length, even more preferably at least 30 nucleotides in length, still more preferably at least 40 nucleotides in length and even still more preferably at least 50 nucleotides in length.

15

The nucleic acid template molecule may be in a recombinant form, in a virus particle, bacteriophage particle, yeast cell, animal cell, or a plant cell. Preferably, the nucleic acid template molecule is derived from a human or laboratory animal species.

20   Those skilled in the art will be aware that there are many known variations of the basic polymerase chain reaction procedure (McPherson et al, 1991). The present invention extends to the use of all such variations in the isolation of Ox receptor-encoding genetic sequences.

25   The isolated nucleic acid molecule which encodes the Ox receptor polypeptide or a functional part thereof may be cloned into a plasmid or bacteriophage molecule, such as for the production of recombinant gene products. Methods for the production of such recombinant plasmids, cosmids, bacteriophage molecules or other recombinant molecules are well-known to those of ordinary skill in the art and can be accomplished without

undue experimentation. Accordingly, the invention further extends to any recombinant plasmid, bacteriophage, cosmid or other recombinant molecule comprising the nucleic acid molecules described herein.

5   Still yet another aspect of the invention contemplates a method of identifying a modulator of Ox receptor activity, said method comprising assaying recombinant Ox receptor activity in the presence of a potential modulator and comparing said activity to the activity of recombinant Ox receptor in the absence of said potential modulator.

10   As used herein, the term "modulator" shall be taken to refer to any chemical compound, molecule or macromolecule which is capable of altering the activity of an Ox receptor polypeptide, in particular a mammalian Ox receptor polypeptide, including both agonists and antagonists of said activity.

15   Preferably, the subject method further comprises the first step of expressing a functional recombinant Ox receptor polypeptide polypeptide in a cell for a time and under conditions sufficient for said polypeptide to be produced in an assayable quantity.

     The term "assayable quantity" refers to a level of expression of a recombinant polypeptide
20   which is sufficient for the activity of said polypeptide to be determined by any standard assay procedure which is specific for the function of the recombinant polypeptide.

     A further aspect of the invention contemplates a modulator of Ox receptor activity, such as a competitive or non-competitive inhibitor, a competitive or non-competitive activator
25   molecule or a molecule which binds irreversibly to an Ox receptor without leading to activation thereof or alternatively, an antibody molecule or catalytic antibody molecule capable of binding and inhibiting the activity of a Ox receptor polypeptide, amongst others.

- 26 -

Preferably, the modulator is one which is identified using the method described *supra* for the identification of modulators of Ox receptor activity.

The modulator molecules described herein are useful in a wide range of prophylactic and

5   therapeutic applications, by virtue of their ability to agonise or antagonise Ox receptor activity. Antagonists are particularly useful in the treatment of physiological or medical conditions wherein Ox receptor activity is elevated or it is desirable to reduce Ox receptor activity, whilst agonists are useful in the treatment of any conditions associated with reduced Ox receptor activity or where it is desirable to increase Ox receptor activity.

10

In order to facilitate an understanding of the invention reference will be made to the following examples which illustrate some preferred embodiments of the invention and demonstrate that the Ox receptor is a new receptor, distinct from I_2 receptors and α_2-adrenoceptors. However it is to be understood that the particularity of the following

15   description is not to supersede the generality of the preceding description of the invention.

Example 1

    a)   **1-Dicyclohexylmethylamino-3-chloroethyl urea:** Chloroethyl isocyanate (11g in

20   16 ml of tetrahydrofuran) is added dropwise into a solution of dicyclohexylmethylamine (19.5g in 125ml of tetrahydrofuran). After 2hrs the precipitated solid is filtered, washed with tetrahydrofuran and air-dried. Yield= 23.4g, 78%.

25   b)   **2-(Dicyclohexylmethylamino)-oxazoline) (O503):** 1-Dicyclohexylmethylamino-3-chloroethyl urea, 36.1g, finely powdered is suspended in 75ml water. Sufficient tetrahydrofuran is then added to dissolve the solid followed by a 10% solution of aqueous sodium hydroxide (26ml). After refluxing for 1hr the solvent is removed under reduced pressure. The residue is extracted with ethyl acetate. The extracts

30   are washed with brine, dried over sodium sulfate and evaporated to dryness. The

- 27 -

residue is recrystallised from ethyl acetate:hexane. m.p. = 135-138°C Yield = 9.45g, 59%. Proton nmr spectrum in deuterated chloroform shows δ 0.6-2.0 (22H broad doublet, cyclohexyl protons), δ 3.2-4.2 (5H m, oxazoline and methine protons), δ 4.7 (1H s, NH). Chemical ionisation mass spectrum shows MH+ ion

5   at m/e 265.

Example 2

10   a)   **(R)-(-)-1-(1-Cyclohexylethyl)-3-chloroethyl urea:** Chloroethyl isocyanate (11g in 16 ml of tetrahydrofuran) is added dropwise into a solution of (R)-(-)-1-(1-cyclohexylethylamine (12.7g in 125ml of tetrahydrofuran). After 2hrs the precipitated solid is filtered, washed with tetrahydrofuran and air-dried. Yield = 15.3g, 75%. m.p. = 109-113°C.

15 b)   **(R)-(-)-2-(1-Cyclohexylethylamino)-oxazoline) ((R)-(-)-O504) :** (R)-(-)-1-(1-Cyclohexylethyl)-3-chloroethyl urea, 13.7g, finely powdered is suspended in 75ml water. Sufficient tetrahydrofuran is then added to dissolve the solid followed by a 10% solution of aqueous sodium hydroxide (26ml). After refluxing for 1hr the solvent is removed under reduced pressure. The residue is extracted with ethyl

20   acetate. The extracts are washed with brine, dried over sodium sulfate and evaporated to dryness. The residue is recrystallised from ethyl acetate:hexane, m.p. = 76-79°C. Yield = 7.6g, 58%. Proton nmr spectrum in deuterated chloroform shows δ 0.9-1.9 (11H bd, cyclohexyl protons), δ 1.07 (3H d, methyl protons), δ 3.5-4.3 (4H m, oxazoline protons), δ 3.4 (1H q, methine proton), δ 4.5 (1H s,

25   NH). Chemical ionisation mass spectrum shows MH+ ion at m/e 197.

Example 3

        a)   **(S)-(+)-1-(1-Cyclohexylethyl)-3-chloroethyl urea:** Chloroethyl isocyanate (11g

30   in 16 ml of tetrahydrofuran) is added dropwise into a solution of (S)-(+)-1-(1-

cyclohexylethylamine (12.7g in 125ml of tetrahydrofuran). After 2hrs the precipitated solid is filtered, washed with tetrahydrofuran and air-dried. Yield = 15.5g, 76%, m.p. = 107-110°C.

5   b)   **(S)-(+)-2-(1-Cyclohexylethylamino)-oxazoline ((S)-(+)-O504)** : (S)-(+)-1-(1-Cyclohexylethyl)-3-chloroethyl urea, 13.7g, finely powdered is suspended in 75ml water. Sufficient tetrahydrofuran is then added to dissolve the solid followed by a 10% solution of aqueous sodium hydroxide (26ml). After refluxing for 1hr the solvent is removed under reduced pressure. The residue is extracted with ethyl

10   acetate. The extracts are washed with brine, dried over sodium sulfate and evaporated to dryness. The residue is recrystallised from ethyl acetate:hexane, m.p. = 70-75°C. Yield = 7.4g, 56%. Proton nmr spectrum in deuterated chloroform shows δ 0.9-2.0 (11H bd, cyclohexyl protons), δ 1.1 (3H d, methyl protons), δ 3.5-4.2 (4H m, oxazoline protons), δ 3.4 (1H q, methine proton), δ 4.5 (1H s,

15   NH). Chemical ionisation mass spectrum shows MH+ ion at m/e 197.

Example 4

Receptor Affinities and Selectivities of Selected Drugs for Ox receptors, α_2-adrenoceptors and I_2-receptors in Rat Brain Homogenates

20

The following compounds were tested for their affinities and selectivities for Ox, I_2 and α_2-receptors in brain homogenates.

25

30

Compound	Formula*	R_1	R_2	R_3	R_4	R_8	R_9	R_{10}
Rilmenidine	II	cyclopropyl	cyclopropyl	-	-	-	-	-
BAY-A6781	III	-	-	methyl	ethyl	-	-	-
(+)-S8349	II	CF_3	cyclopropyl	-	-	-	-	-
(-)-S8350	II	CF_3	cyclopropyl	-	-	-	-	-
O501	III	-	-	ethyl	ethyl	-	-	-
O502	II	phenyl	phenyl	-	-	-	-	-
O503	VI	-	-	-	-	H	H	-
(S)-(+)-O504	VII	-	-	-	-	-	-	methyl
(R)-(-)O504	VII	-	-	-	-	-	-	methyl
(S)-(-)-O506	II	methyl	1-naphthyl	-	-	-	-	-

*where X is O, Y is $-CH_2CH_2-$, Z is NH and, for formula II, W is $-CHR^1R^2$, and for formulae

III, VI and VII R^5 and R^6 are H.

Methoxyidazoxan is 2-(2-methoxy-1,4-benzodioxan)-imidazoline); clonidine is 2-(2,6-dichlorophenylamino)-imidazoline; idazoxan is 2-(1,4-benzodioxan)-imidazoline; cirazoline is 2-(2-cyclopropylphenoxymethyl)-imidazoline; BU-224 is 2-(4,5-dihydroimidaz-2-yl)quinoline.

The results are shown below in Tables 1 and 2.

Table 1

Compound	Receptor Affinities (nM)		
	Ox	I_2	Selectivity Ox/I_2[*]
Methoxyidazoxan	319,000	24,400	0.08
Clonidine	7030	5270	0.7
Idazoxan	62,300	2.3	0.00004
Cirazoline	21,200	1.6	0.00008
BU-224	>100,000	0.9	<0.000009
Rilmenidine	15	36	2.4
O501	18	6,900	383
O502	160	1,900	12
O503	117	3,800	32
(S)-(+)-O504	106	707	6.7
(R)-(-)-O504	31	56	1.8

[*]Compounds more selective for the Ox site have a selectivity > 1

Table 2

Compound	Receptor Affinities (nM)		
	Ox ·	α_2	Selectivity Ox/ α_2*
Methoxyidazoxan	319,000	1.9	0.000006
Clonidine	7030	2.7	0.0004
Idazoxan	62,300	15.6	0.0003
Cirazoline	21,200	2.6	0.0001
BU-224	>100,000	5,477	<0.1
Rilmenidine	15	410	27
O501	18	210	12
O502	160	380	2.4
O503	117	640	5.5
(S)-(+)-O504	20	30	1.5
(R)-(-)-O504	32	590	18

*Compounds more selective for the Ox site have selectivity of > 1

The affinity and selectivity of the claimed compounds in the rat is exemplified by comparisons with conventional ligands in Tables 1 and 2, in which selectivity is defined as the reciprocal of the ratio of the affinities in nanomolar concentrations for each site.

In the rat, selective α_2-adrenoceptor antagonists such as methoxyidazoxan exhibit poor affinity (μM) for both the Ox receptor and the I_2-receptor and high affinity (nM) for the α_2-adrenoceptor. Such a binding pattern indicates that the Ox receptor and I_2-receptors are distinct sites from the α_2-adrenoceptors. Clonidine, which has been reported widely to be an I_1 and α_2-receptor ligand, also displays poor affinity (μM) for both the Ox receptor and I_2-receptor and high affinity (nM) for α_2-adrenoceptors, indicating that both Ox receptors and I_2-receptors are binding sites distinct from the I_1- and α_2-receptors. Known I_2-receptor ligands, such as idazoxan, cirazoline and BU-224, display poor (μM) affinity for the Ox

- 32 -

receptor and high affinity (nM) for the I_2-receptor, consistent with being highly selective I_2 ligands. This indicates that the Ox receptor and I_2-receptor are distinct. The I_2-receptor ligands idazoxan and cirazoline also have a high affinity (nM) for α_2-adrenoceptors, whereas BU-224 has poor affinity (μM) for this receptor. The oxazoline rilmenidine is a known α_2-

5 adrenoceptor agonist which has a high affinity (nM) for both the Ox receptor and the I_2-receptor in the rat but lower affinity in human brains.

Example 5

10 **Receptor Affinities and Selectivities of Selected Drugs for Ox receptors and I_2-receptors in Human Brain Homogenates.**

The following compounds were also tested for their affinities and selectivities for Ox receptors and I_2- receptors in human brain homogenates. The results are shown in table 3.

15

Table 3

Compound	Receptor Affinities (nM)		
	Ox	**I_2**	**Selectivity Ox/I_2**
Methoxyidazoxan	100,000	>100,000	1
Clonidine	17,930	18,460	1.0
Idazoxan	6060	4.8	0.0008
Cirazoline	1074	1.6	0.0015
BU-224	9576	2.2	0.0002
BAY-A6781	215	8,250	38
Rilmenidine	396	5,220	13
(+)-S8349	268	13,000	49
(-)-S8350	199	7,700	32
O501	38	9,100	239
O502	234	686	3

O503	70	4,800	69
(S)-(+)-O504	180	2,190	12
(R)-(-)-O504	300	2,700	9
(S)-(-)-O506	99	7,580	77

5 *Compounds with selectivity > 1 show significant selectivity for the Ox receptor.

In humans, all of the oxazolines tested displayed significant selectivity (3-239) for the Ox receptor over the I_2-receptor.

10 Measurement of the affinity and selectivity of such compounds for the α_2-adrenoceptor, I_2-receptor and Ox receptor binding sites may be carried out by either of two procedures, i.e. ligand displacement studies in membranes of brain and kidney or by autoradiography of brain and kidney sections. In both cases the radioligand is tritiated rilmenidine ([^{3}H]-RIL). Rilmenidine is an oxazoline compound, which is an effective anti-hypertensive agent and

15 originally believed to act as a central and peripheral α_2-adrenoceptor agonist. As rilmenidine not only binds to α_2-adrenoceptor sites but also in the rat to I-receptors, Ox receptor sites may be preferentially labelled by incubation with [^{3}H]-RIL in the presence of μM concentrations of α_2-adrenoceptor ligands such as adrenaline or methoxyidazoxan with or without the addition of the I_1- and I_2-receptor ligands clonidine and idazoxan. Analysis of the

20 displacement of [^{3}H]-RIL from these sites by drugs reveals the affinity of the drug for each binding site.

RECEPTOR BINDING STUDIES

Brain Membrane preparation

25 Male WKY rats are stunned and decapitated. The brains are removed and the forebrain dissected free, then frozen over liquid nitrogen before being stored at -70°C. Human brain cortex samples are obtained within 28 hours of death and frozen over liquid nitrogen before being stored at -70°C. On the day of the measurement the cortex (human or rat) is homogenised in 20 vol ice-cold 50 mM Tris.HCl buffer (pH 7.4) using a Polytron tissue

30 homogeniser. The homogenate is centrifuged at 37000 x g for 10 min and the pellet

resuspended in 20 vol of fresh buffer and recentrifuged. The pellet is then resuspended in 33 vol of 50mM Tris.HCl buffer for use in binding assays. Where adrenaline is used in binding assays the buffer also contains 25µM EDTA and 10 µM pargyline.

5   **Measurement of Ox receptor binding sites**

Drug inhibition of [^{3}H]-RIL (38-49 Ci/mmol) binding is measured in duplicate in samples containing 5nM [^{3}H]-RIL and competing drug 0.01nM to 10mM, 10µM adrenaline and 15mg of membranes in a final volume of 1ml. After incubation for 30 min at 25°C, the membranes are recovered by filtration onto Whatman GF/B glass fibre filters presoaked in

10   1.0% polyethyleneimine. The filters are washed 3 times with cold assay buffer before measurement of bound radioactivity by scintillation counting. The binding data are analysed by an iterative non-linear computerised curve fitting program "RADLIG" version 4.0 (McPherson, 1983).

15   **Measurement of α$_2$-adrenoceptor and I$_2$-receptor binding sites**

Brain membranes prepared as above, are incubated in the presence of [^{3}H]-methoxyidazoxan (52 - 57 Ci/mmol) 0.5nM or [^{3}H]-idazoxan (43-45Ci/mmol) 1.6nM in Tris.HCl buffer pH 7.4 for α$_2$-adrenoceptor and I$_2$-receptor binding respectively. Inhibition of binding is measured in the presence of increasing amounts of test compound (0.1nM to 1mM). Non-specific

20   binding is estimated by addition of 10µM phentolamine or 100µM cirazoline respectively. Membrane bound radioactivity is isolated and measured as above by vacuum filtration, washing and scintillation counting. Data is analysed by the "RADLIG" program.

AUTORADIOGRAPHIC STUDIES

25   **Preparation of tissue sections**

Male WKY rats are stunned and decapitated. The brains, kidneys or spinal cords are rapidly excised , frozen over liquid nitrogen and stored at -70°C until used. The tissues are then mounted onto microtome chucks with Tissue-Tek embedding matrix. Horizontal, coronal or sagittal sections are cut (10 to 14µm thick) at -15°C and thaw mounted onto cold

30   gelatine/chrome alum coated slides. 2 to 4 sections are mounted on each slide, air-dried at

room temperature, then stored with silica gel dessicant at -70°C up to 2 weeks before use.

Measurement of Ox receptor binding sites in tissue sections

The mounted sections are brought to room temperature and preincubated for 20 min in

5   50mM Tris.HCl buffer (pH 7.6) to remove endogenous inhibitors such as noradrenaline. The slides are then dried at room temperature. Each section is then covered with the same buffer containing 40nM [^{3}H]-RIL ± 1μM adrenaline or 10μM of the oxazoline BAY A6781 and incubated at room temperature for 60 min to assess total, non-adrenergic and non-specific binding respectively. Breakdown of adrenaline is minimised by addition of 25μM EDTA and

10   10μM pargyline. The incubations are terminated by washing the sections in fresh buffer for 4 min and rinsing in distilled water. Sections are then air-dried and desiccated overnight. The sections are then apposed to tritium sensitive (Hyperfilm™ -^{3}H, Amersham) film in X-ray cassettes for 4 to 16 weeks at 4°C. After exposure the films are developed in Kodak D19 at 18 to 20°C for 5 min. Autoradiograms are analysed by computer aided densitometry and

15   calibrated by co-exposed tritium standards.

Example 6

Method of isolating and sequencing the Oxazoline receptor

20   A tissue rich in Ox receptors, such as rabbit kidney, is homogenised in 10 mM Tris buffer containing 5 mM EGTA, 5 mM EDTA and 10μM phenylmethylsulfonyl fluoride pH7.4 at 4°C. The homogenate is subjected to differential centrifugation to isolate the plasma membrane fraction. The protein is solubilised with buffer containing octylglucoside detergent and purified by affinity chromatography with a column of sepharose coupled to a ligand such

25   as formula II where X is O, Y is -CHR^{5}CHR^6- where R^5 and R^6 are H, W is -CHR^{1}R^2 wherein R$_1$ is 4-amino,2-methoxyphenyl, R$_2$ is H and Z is NH. Binding of α$_2$-adrenoceptors and I-receptors to the column can be minimised by the addition of methoxyidazoxan and 2-BFI, and the Ox-receptor selectively eluted with buffer containing O501. The eluate is then dialysed against drug-free buffer to remove O501. The Ox receptors in the eluate are

30   selectively labelled with a photoaffinity label such as (^{125}I)-AZIPI (Lanier et al, 1995) or

related compounds derived from formula II where X is O, Y is -CHR^{5}CHR^6- where R^5 and R^6 are H, W is -CHR^{1}R^2 wherein R^1 is 4-amino, 2-methoxyphenyl, R$_2$ is H and Z is NH, by exposure to ultraviolet light. The labelled Ox receptors are then precipitated with trichloroacetic acid, washed with ether and solubilised in Laemmli loading buffer and

5   electrophoresed on 10% polyacrylamide gel under denaturing conditions. After electrophoresis, the gels are dried under vacuum and exposed to X-ray film for 1 to 8 days. The labelled bands can be located after development of the X-ray films and transferred electrophoretically to polyvinylidene difluoride membranes. The areas corresponding to the labelled Ox receptors are cut out and digested with trypsin. The generated peptides can then

10 be separated by microbore reversed phase high performance liquid chromatography and the amino acid sequence of each determined. These sequences can then be used as the basis for generation of oligonucleotide probes for the dna sequence of the Ox receptor.

Those skilled in the art will appreciate that the invention described herein is susceptible to

15 variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps of features.

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- 37 -

REFERENCES

Alemany R, Olmos G, Escriba PV, Menargues A, Obach R and Garcia-Sevilla JA. LSL
60101, a selective ligand for imidazoline I-2 receptors, on glial fibrillary acidic protein
5 concentration. Eur. J. Pharmacol. 1995; 280, 205-210.

Evans RG Current status of putative imidazoline (I_1) receptors and renal mechanisms in
relation to their antihypertensive therapeutic potential. Clin. Exp. Pharmacol. Physiol. 1996;
23: 845-854.
10

Gustafson I, Westerberg, E. and Wieloch, T. Protection against ischemia-induced neuronal
damage by α_2-adrenoceptor antagonist idazoxan: influence of time of administration and
possible mechanisms of action. J. Cereb. Blood Flow Metab. 1990; 10, 885-894.

15 Hosseini R, King PR, Louis WJ and Gundlach AL. [^{3}H]2-(2-benzofuranyl)-2-imidazoline, a
highly selective radioligand for I_2-imidazoline receptor binding sites: Studies in rabbit kidney
membranes. Naun.-Schmied. Arch. Pharmacol. 1997; 355:131-138.

King PR, Gundlach AL and Louis WJ. Quantitative autoradiographic localization in rat brain
20 of α_2-adrenoceptors and non-adrenergic I-receptor binding sites labelled by [3H] rilmenidine.
Brain Res. 1995; 675, 264-278.

Lanier B, Raddatz R, Bakthavachalam V, Coupry I, Neumeyer JL and Lanier SM.
Structural and Ligand Recognition Properties of Imidazoline Binding Proteins in Tissues of
25 Rat and Rabbit. Mol. Pharmacol. 1995; 48: 703-710.

Lione LA, Nutt DJ and Hudson L. [^{3}H]2-(2-Benzofuranyl)-2-imidazoline: a new selective
high affinity radioligand for the study of rabbit brain imidazoline I_2 receptors. Eur. J.
Pharmacol. 1996; 304, 221-229.
30

Mallard NJ, Hudson AL and Nutt DJ Characterization and autoradiographical localization of non-adrenoceptor idazoxan binding sites in the rat brain. Br. J. Pharmacol. 1992; 106, 1019-1027.

5 McPherson, GA A practical computer-based approach to the analysis of radioligand binding experiments. Comput. Programs Biomed. 1983; 17: 107-113.

McPherson, M.J., Quirke, P. and Taylor, G.R. (1991) PCR A Practical Approach. IRL Press, Oxford University Press, Oxford, United Kingdom.

10

Nero TL, Hosseini AR, King PR, Iakovidis D, Gundlach AL and Louis WJ. Development of an I_2-receptor pharmacophore, presented at the International Workshop on Imidazoline Receptors, September 10, 1997, Melbourne, Victoria, Australia (Satellite Meeting to the International Society of Autonomic Neurosciences 1997), p44 of the conference handbook.

15

Olmos G, Alemany R, Escriba PV and Garcia-Sevilla JA. The effects of chronic imidazoline drug treatment on glial fibrillary protein concentrations in rat brain. Br. J. Pharmacol. 1994; 111, 997-1002.

20 Parini A, Moudanos CG, Pizzinat N and Lanier SM. The Elusive Family of Imidazoline Binding Sites. TIPS 1996; 17: 13-16.

Petito CK, Morgello S, Felix JC and Lesser ML. The two patterns of reactive astrocytosis in postischemic rat brain. J. Cereb. Blood Flow Metab. 1990: 10, 850-859.

25

Regunathan S, Feinstein DL and Reis DJ. Expression of non-adrenergic imidazoline sites in rat cerebral cortical astrocytes. Neurosci. Res. 1993; 34: 681-688.

Regunathan S and Reis DJ. Imidazoline receptors and their endogenous ligands. Annu. Rev.

30 Pharmac. Toxicol. 1996; 36: 51-44.

Sastre M and Garcia-Sevilla JA. Opposite age-dependent changes of α_{2A}-adrenoceptors and [^{3}H]Idazoxan binding sites (I_2-imidazoline sites) in the human brain: strong correlation of I_2 with MAO-B sites. J. Neurochem. 1993; 61: 881-889.

5   Tesson F, Limon-Boulez I, Urban P, Puype M, Vandekerckhove J, Coupry I, Pompou D and Parini A. Localisation of I_2-Imidazoline Binding Sites on Monoamine Oxidases. J. Biol. Chem. 1995; 270: 9856-9861.

Timmermans, PBMWM and van Zwieten PA. QSAR in centrally acting imidazolines
10 structurally related to clonidine. J. Med. Chem. 1977; 20: 1636-1645.

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